PLANTS WITH CONTROLLED SIDE-SHOOT FORMATION AND/OR ABSCISSION ZONE FORMATION

The present invention relates to nucleotide sequences encoding polypeptides which are responsible for controlling side-shoot formation and/or petal formation and/or abscission zone formation as well as to the polypeptides and amino acid sequences encoded by said nucleotide sequences. Furthermore, the present invention relates to plants having controlled side-shoot formation and/or petal formation and/or controlled formation of abscission zones, wherein the expressible DNA sequence or fragment or derivative thereof responsible for side-shoot formation and/or petal formation and/or abscission zone formation is integrated in a stable manner into the genome of the plant cell or the plant tissue. Further, the invention relates to methods for the production of plants having controlled side-shoot formation and/or petal formation and/or controlled formation of abscission zones, wherein the expressible DNA sequence or fragment or derivative thereof responsible for side-shoot formation and/or petal formation and/or abscission zone formation is integrated in a stable manner into the genome of plant cells or plant tissues and the resulting plant cells or plant tissues are regenerated to form plants. Moreover, the invention relates to plants and seed stocks of plants, which are obtainable according to the method of the invention.

Technical Background

The performance characteristics of economic and ornamental plants are considerably determined by their architecture. While the basic structure of a plant manifests in the embryonic development, the post-embryonic phase is characterized by the activity of apical meristems. Of fundamental importance is the ability of the shoot apical meristem (SAM) of higher plants to initiate shoot branches and to control their development. As a result, the habit of a plant and thus an essential performance feature is characterized by the number, arrangement and developmental intensity of its side-shoots. The branching of the shoot may occur terminally as well as laterally. The terminal branching in which the SAM is separated into two portions mainly occurs in lower cormophytes and has been described for only a few flowering plants (Steeves and Sussex, 1989, Patterns in Plant Development, 2nd Edition, Cambridge University Press,

Cambridge). The lateral branching typical for flowering plants is based on the formation of new shoot apical meristems in the leaf axils, which are derived from SAM cells, the meristemic character of which remains preserved in contrast to surrounding cells which are involved in the development of leaf primordia. In the further course of development, a side bud is formed from said residual meristems, which besides some leaf primordia contains an apical meristem, the activity of which is subject to the control by the main shoot SAM.

The analysis of plant mutants revealed that branching of the shoot system is controlled by genetic factors. Thus, in tomato (*Lycopersicon esculentum*) for example, there have been described a number of mutants, the side-shoot formation of which is inhibited in different stages (e.g. *blind, blind-2, torosa, lateral suppressor*). A morphological characterization showed that the production of axil buds is disturbed in the tomato mutants *blind, blind-2* and *torosa* (Tucker, 1979, Ann. Bot. 43: 571-577; Mapelli and Lombardi, 1982, Plant & Cell Physiol. 23: 751-757). In contrast, in plants which are homozygous for recessive *lateral suppressor* (*ls*) mutation, the initiation of most of the side buds does not occur (Brown, 1955, Rep. Tomato Genetics Cooperative 5: 6-7). A histological analysis (Malayer and Guard, 1964, Amer. Jour. Bot. 51: 140-143) shows that cells directly derived from SAM in the axils of the leaf primordia, on the meristemic activity of which the formation of side shoots is based, are missing in the *lateral suppressor* mutant. If a lack of side shoots in all leaf axils results in a termination of the shoot axis in the first inflorescence, the transition to floral development shows that the ability to establish axil meristems is not completely lost in the mutant. In the axil of the leaf primordium established directly before the inflorescence a meristem often is established in homozygous *ls* mutants as well. The establishment of this meristem which is necessary for the sympodial structure of the shoot axis is often associated by the formation of a side bud in the axil of the next older leaf. Following the transition to the floral phase, the development of the *ls* mutant is characterized by a smaller number of flowers per inflorescence (Williams, 1960, Heredity, 14: 285-296), the missing establishment of petal primordia (Szymkowiak and Sussex, 1993, Plant J., 4: 1-7) and an aberrant number of stamens and carpels (Groot *et al.*, 1994, Sci. Hort., 59: 157-162). Furthermore, a reduced fertility in the mutant is observed, which also

results in the reduction of yield and which is the reason that the *ls* mutant did not reach any significance for yield-oriented cultivation.

A further phenotypic change of the *ls* mutant relates to the formation of abscission zones in the flower and fruit stems. While wild type plants have a region of 5-10 layers of smaller cells, at the distal ends of which the non-pollinated flower or the ripe fruit comes off the plant (Roberts *et al.*, 1984, Planta, 160: 159-163), this abscission zone is not formed in the *ls* mutant and during harvest the fruit comes off the plant without residues of the fruit stem and sepals.

The observed phenotypic changes are correlated with disorders in the equilibria of particular plant hormones on a physiological level. In comparison with the wild type, lower cytokinin concentrations were measured in the shoot tips of *ls* mutants (Maldiney *et al.*, 1986, Physiol. Plant, 68: 426-430; Sossountzov *et al.*, 1988, Planta, 175: 291-304), while the amounts of β-indolylacetic acid (IAA)-like compounds as well as gibberellic and abscisic acids are markedly increased (Tucker, 1976, New Phytol., 77: 561-568). Attempts to remedy the deficiencies of the *ls* mutant by introducing an isopentenyl transferase gene from *Agrobacterium tumefaciens* resulted in an increase of endogenous cytokinine concentrations, but not in a normalization of the side-shoot development (Groot *et al.*, 1995, Plant Growth Regulation, 16, 27-36).

Due to the great interest of breeders in single stem tomato varieties there have been early efforts to render the *ls* mutant usable for commercial cultivation. Since the DNA sequence of the gene (*Ls* gene) responsible for side-shoot formation and/or petal formation and/or abscission zone formation has so far not been known, it was repeatedly attempted by genetic methods to separate the desired effects on the side-shoot formation from the non-desired effects on fertility and yield. However, up to now none of these efforts have been successful.

For the isolation of genes which are only characterized by a mutant phenotype and their position on the genetic map, the strategies of insertional mutagenesis and positional cloning have been preferably used during the past years. The insertional mutagenesis uses mutant alleles formed by the insertion of a known sequence for the isolation of genes which in this manner are labeled on a molecular level. In plants, the T-DNA from *Agrobacterium tumefaciens* (Koncz *et al.*, 1992, Plant Mol. Biol., 20: 963-976) as well as transposable elements (Gierl and Saedler, 1992, Plant Mol. Biol., 19; 39-

49) were used for insertional mutagenesis (Jones *et al.*, 1994, Science 266: 789-793). Since the transposable elements *Ac* and *Ds* from maize preferentially transpose to coupled positions on the same chromosome (Knapp *et al.*, 1994, Mol. Gen. Genet., 243: 666-673) a transposon mutagenesis is particularly promising when a starting line is available in which the transposable element is present in close coupling with the gene of interest. Since such a tomato line is not available, a transposon mutagenesis for the isolation of the *Ls* gene is not very promising.

The strategy for positional cloning was developed for the analysis of the molecular principles of hereditary diseases in mammals and *inter alia* used for the isolation of human genes for Duchenne's muscular dystrophy (Koenig *et al.*, 1987, Cell, 50: 509-517), Cystic Fibrosis (Rommens *et al.*, 1989, Science, 245: 1059-1065) and Huntington's Disease (Huntington's Disease Research Group, 1993, Cell 72: 971-983). Figure 1 schematically illustrates the course of a positional cloning. For this strategy the integration of the classical genetic locus into a map of molecular markers is of fundamental importance. The use of restriction fragment length polymorphisms (RFLPs) as genetic markers (Botstein et al., 1980, Am. J. Hum. Genet., 32: 314-331) enables the identification of closely coupled DNA fragments from the environment of the gene to be isolated. These fragments subsequently serve as hybridizing probes in Southern analysis by means of pulsed field gel electrophoresis (Chu *et al.*, 1986, Science, 234, 1582-1585) of separated high molecular weight DNA to transform the relative genetic distance into an absolute value for the physical distance which has to be bridged by the so-called "chromosome walk". Starting with flanking markers as starting points the environment of the desired gene is isolated in the form of overlapping DNA fragments. Depending on the distance of the flanking markers in the genetic map the DNA fragments are YAC or cosmid clones (Burke *et al.*, 1987, Science, 236: 806-812). RFLP maps with high marker density have been developed by Nam *et al.*, 1989, Plant Cell, 1, 699-705, and Tanksley *et al.*, 1992, Genetics, 132: 1141-1160. Grill and Somerville, 1991, Mol. Gen. Genet., 226: 484-490, and Martin *et al.*, 1992, Mol. Gen. Genet, 233: 25-32, describe the preparation of YAC-libraries.

In the classical genetic map of tomato the *Ls* locus is mapped on the long arm of chromosome 7 (Taylor and Rossall, 1982, Planta, 154: 1-5). Schumacher *et al.*, 1995, Mol. Gen. Genet, 246: 761-766, describe an integration of the *Ls* locus into the RFLP

map, wherein the *Ls* locus was mapped within a 0.8 cM interval near the distal end of chromosome 7. Furthermore, Schumacher *et al.* describe that the *Ls* locus is bounded by the RFLP markers CD61 and CD65. The physical mapping by means of pulsed field gel electrophoresis showed that CD61 and CD65 are not more than 375 kb apart from each other.

With respect to agricultural cultivation the formation of side shoots is not desired in many economic plants due to various reasons:

1. Firstly, the young side shoots are "sink" organs (organs of consumption) and thus reduce the yield of the main shoot.

2. Highly branched shoot systems often represent a hardly surmountable obstacle for mechanical treatment (e.g. harvest with machines).

For these reasons there have been early attempts to cultivate varieties without side shoots in a conventional manner. This has been successful in individual economic plants (e.g. sun flower). However, in many other dicotyledonous economic plants (e.g. tomato, cucumber, apple-tree, pear-tree) the single stem would be desirable, but this has so far not been realized in efficient culture varieties. Also in monocotyledonous economic plants, such as maize and sugar cane, suppression of side shoot formation is advantageous and highly desired for commercial use. At present, the single stem e.g. of tomato is achieved in green house cultivation common in Central and Northern Europe by manually removing the side shoots. Since the removal of the side shoots cannot be done with machines this is associated with enormous costs. Furthermore, at the wound site the plants are very susceptible of infections by pathogens, such as pathogenic bacteria, viruses and fungi. Thus, the removal of side shoots contributes to the spreading of diseases in green house.

In many ornamental plants, however, the additional formation of side shoots and thus an enhanced formation of flowers is desired. Enhanced formation of side shoots is also highly beneficial in many economic plants, such as potato, coffee or tea plant. Thus, there is a need for cost-effective, efficient economic plants and ornamental plants, in which the formation of side shoots is increased or suppressed.

Inhibition of the formation of abscission zones is of interest in a number of plants. Thus, the premature abscission of fruits in citrus plants resulted in losses of yield which could be prevented if no abscission zones were formed. Similar results may be

found in other fruit species, such as cherry, peach or black currant. Further, an inhibition of the formation of abscission zones, e.g. in tomato, is advantageous. If the abscission zones are not formed, the fruit comes off the plant during harvest without residues of the fruit stem and sepals. This feature is desired when tomatoes are harvested with machines and are subsequently processed to products such as tomato puree, since sepals and fruit stems deteriorate the quality of the tomato products.

In ornamental plants, an increased formation of abscission zones may be useful, since flowers would fall off by themselves after fading and there would be no need to remove them manually, such as with many balcony and garden plants. If this does not occur, the formation of new flowers is suppressed.

Short Description of the Invention

Isolation and cloning of the *Ls* gene would offer the possibility to change the activity of said gene in a targeted manner and thus to suppress or increase the formation of side shoots in transgenic plants. Further, one may suppress or increase the formation of abscission zones and/or petals by changing the activity of the *Ls* gene in a targeted manner. Accordingly, the object underlying the present invention is to isolate the *Ls* gene or a DNA fragment containing said gene, determine its sequence and provide a method for the preparation of transgenic plants in which the activity of the *Ls* gene was varied in a targeted manner to suppress or increase the formation of side shoots and/or the formation of abscission zones and/or petals.

The object of the present invention is solved by providing the nucleotide sequences according to SEQ ID NO: 1, 9 or 13 and the nucleotide sequences hybridizing to the nucleotide sequence according to SEQ ID NO: 1, 9 or 13, wherein said nucleotide sequences according to SEQ ID NO: 1, 9 or 13 and said nucleotide sequences hybridizing to the nucleotide sequence according to SEQ ID NO: 1, 9 or 13 encode polypeptides which are responsible for controlling side-shoot formation and/or petal formation and/or abscission zone formation. According to the present invention, the term "hybridization" is directed to conventional hybridization conditions, preferably "hybridization" is directed to such hybridization conditions in which the T_M value is in the range from T_M 45°C to T_M 68°C. The term "hybridization" is particularly preferably

directed to stringent hybridization conditions. The invention further relates to polypeptide and amino acid sequences encoded by said nucleotide sequences.

A further object of the invention is solved by a method for preparing plants having controlled side-shoot formation and/or petal formation and/or abscission zone formation, wherein the expressible DNA sequence or fragment or derivative thereof responsible for controlling side-shoot formation and/or petal formation and/or abscission zone formation is integrated in a stable manner into the genome of plant cells or plant tissues and the resulting plant cells or plant tissues are regenerated to form plants.

In the present invention a method is preferred in which the integrated DNA suppresses the side-shoot formation and/or petal formation and/or abscission zone formation. Particularly preferred is a method in which the integrated DNA is expressed in an antisense orientation with respect to the complementary endogenous sequence controlling side-shoot formation and/or petal formation and/or abscission zone formation. Also particularly preferred is a method in which the integrated DNA is expressed in a sense orientation with respect to the complementary endogenous sequence controlling side-shoot formation and/or petal formation and/or abscission zone formation. Furthermore, particularly preferred is a method in which side-shoot formation and/or petal formation and/or abscission zone formation is suppressed by a ribozyme comprising the DNA sequences or fragment or derivative thereof according to the present invention. Particularly preferred is also a method in which the DNA sequences or fragment or derivative thereof according to the invention are used to switch off ("knock-out") the endogenous gene in plants by way of homologous recombination.

In the present invention a method is further preferred wherein the DNA integrated into the genome of the plants enhances side-shoot formation and/or petal formation and/or abscission zone formation. Particularly preferred is a method in which the DNA according to the invention is expressed in a sense orientation with respect to the endogenous sequence responsible for side-shoot formation and/or petal formation and/or abscission zone formation.

Particularly preferred is the method according to the invention for the preparation of transgenic tomato, rape, potato or snapdragon plants. Particularly

preferred is also a method according to the present invention for the preparation of transgenic plants, wherein the DNA integrated into the genome of the plants comprises the sequence according to SEQ ID NO: 1, 9 or 13 or fragment or derivative thereof or which is complementary to said sequence or fragment or derivative thereof, or which hybridizes with the sequence according to SEQ ID NO: 1, 9 or 13 or fragment or derivative thereof and encodes a polypeptide having the biological activity of side-shoot formation and/or petal formation and/or abscission zone formation.

The invention further relates to transformed plant cells or transformed plant tissue, wherein an expressible DNA sequence or fragment or derivative thereof responsible for controlling side-shoot formation and/or petal formation and/or abscission zone formation is integrated in a stable manner into the genome of the plant cell or plant tissue. Further, the invention relates to plants as well as to seed stocks of plants obtainable according to the method of the present invention.

The invention is further illustrated by the following figures, wherein:

Figure 1 schematically shows the course of a positional cloning.

Figure 2 illustrates in (a) a portion of the RFLP map published by Tanksley *et al.*, 1992, Genetics, 132: 1141-1160. In (b) the *Ls* region according to Schumacher *et al.*, 1995, Mol. Gen. Genet., 246: 761-766, is integrated into this map.

Figure 3 shows the mapping of cDNA and cosmid clones from the *Ls* region. The cosmid clones A, B, C, D, E, F, G and L as well as YAC clone CD61-5 are symbolized by bars. The positions of the cDNA clones c10, c21, y25 and ET are illustrated by open rectangles. The dashed lines represent recombination sites in F2 plants 23, 24, 865 and 945.

Figure 4 shows the autoradiograph of a Southern blot analysis for the detection of *Ls*-related genes in different plant species. Genomic DNA from tomato (*Lycopersicon esculentum*), potato (*Solanum tuberosum*) and snapdragon (*Antirrhinum majus*) was treated with the restriction enzyme EcoRI and hybridized with the cDNA clone ET.

Figure 5 shows the nucleotide sequence and the amino acid sequence derived therefrom (one letter code) of the *Ls* wild type gene from tomato (*Lycopersicon esculentum*).

Figure 6 shows the nucleotide sequence and amino acid sequence derived therefrom (one letter code) of the *Ls* homologous gene from potato (*Solanum tuberosum*).

Figure 7 shows the nucleotide sequence and the amino acid sequence derived therefrom (one letter code) of a 687 bp DNA fragment of the *Ls* homologous gene from *Arabidopsis thaliana*.

Figure 8 shows an alignment of amino acid sequences of the *Ls* polypeptide from *Arabidopsis thaliana* (LsAt), *Lycopersicon esculentum* (LsLe) and *Solanum tuberosum* (LsSt). The one letter code was used for amino acids. Identical amino acids are shaded in black, similar amino acids are shaded in gray. The dash (-) represents missing sequence information, a dot (.) represents an additional amino acid in a polypeptide. An asterisk (*) represents a stop codon on nucleic acid level.

Detailed Description of the Invention

The method of cloning DNA fragments being several hundreds of kilobases in length as artificial yeast chromosomes (Yeast Artificial Chromosome: YAC) in *Saccharomyces cerevisiae* (Burke *et al.*, 1987, Science, 236: 806-812) enables the transformation of the physical map into a number of overlapping YAC clones spanning the gene to be isolated. From a YAC library of tomato (Martin *et al.*, 1992, Mol. Gen. Genet., 233: 25-32) clones containing the RFLP marker CD61 were isolated. By mapping the YAC terminal fragments with respect to the RFLP markers flanking the *Ls* gene as well as to the recombination break points and to the *Ls* gene itself the position of the isolated DNA fragments in the *Ls* region was determined. Thus, YAC clone CD61-5 was found to hybridize both with CD61 and with CD65 and therefore contains the entire genomic region including the *Ls* gene. Figure 3 schematically illustrates the position of the marker and of the YAC clone.

For identification of coding regions localized within the YAC clone this clone was used as a radiolabeled probe to screen a cDNA library (Simon, 1990, doctoral thesis, University of Cologne, Cologne, Germany). The cDNA library used is made from RNA of both vegetative and floral shoot tips and thus represents expressed genes of the tissues in which the phenotype of the *Ls* mutation manifests itself. A characterization of cDNA clones by cross hybridization revealed that the purified clones

represented a total of 29 different transcripts. The subsequent fine mapping of the cDNA clones relative to the recombination break points in interval CD61-CD65 revealed that only cDNA clone y25 cosegregated with the *Ls* gene and is a possible candidate for said gene. After the establishment of a cosmid contig also cosmid clones were used as probes to isolate further cDNA clones from the CD61-CD65 interval, which in screening with YAC clone CD61-5 as a probe were not detectable due to the high complexity of the probe. In these experiments three additional cDNA clones (c10, c21 and ET) were isolated which also cosegregated with the *Ls* gene and were possible other candidates for the *Ls* gene. Thus, a total of four cDNA clones were identified from the *Ls* region, which were candidates for the *Ls* gene. In Figure 3 said clones are represented by open rectangles.

In order to clone the *Ls* gene together with the promoter sequences necessary for the regulation of expression, the cDNA clone y25 was used as a starting point for the isolation of shorter genomic DNA fragments of the *Ls* region. For this purpose a genomic cosmid library from tomato was established in vector pCLD04541 (Bent *et al.*, 1994, Science, 265: 1856-1860). Said vector contains the T-DNA border sequences necessary for plant transformation and thus allows for an introduction of isolated DNA fragments into plant cells without further cloning steps. From this library a number of overlapping cosmid clones was isolated in several typical cloning steps. Mapping of said cosmid clones relative to the recombination break points in the tested interval showed that the isolated genomic DNA fragments spanned a genomic region of about 60 kb. The position of the cosmid clones is schematically illustrated in Figure 3.

To investigate the question whether a gene from the genomic DNA region isolated as cosmid contig is able to compensate for the biological function for formation of side shoots, petals and abscission zones which is missing in the *ls* mutant (complementation experiment), said *ls* mutant was transformed with the cosmid clones A, B, C, D, E, F, G and L. In all transgenes made by introduction of the cosmids A, B, C, D, E and F, no alteration of the phenotype could be observed. In contrast, in eight independent transgenic plants containing either cosmid G or L a partial or complete recovery of the wild type phenotype could be observed. The results of the complementation experiments are illustrated in Table I.

Cosmid	number of transformed plants	number of complemented plants
pCLDO4541	8	0
A	5	0
B	15	0
C	5	0
D	7	0
E	2	0
F	8	0
G	5	3
L	11	5

Table I: Complementation experiments of *ls* mutant via cosmid transformation

These transgenic plants form side shoots during vegetative development and again petals and abscission zones in the floral development. A Southern blot analysis of transgenic plants containing cosmid G or cosmid L revealed that in plants showing no complementation the T-DNA was only incompletely transferred. Thus, it has been shown that introduced DNA fragments are able to complement the genetic information for formation of side shoots, petals and abscission zones, which is absent from the mutant.

By using complementation experiments with subfragments of cosmid G the DNA region in which the *Ls* gene is localized could be determined in more detail. While following transformation with DNA fragments containing the previously identified gene c21 no complementation of the *ls* phenotype could be observed, the wild type phenotype could be recovered in eight independent transgenic plants by the introduction of an approx. 6 kb fragment bearing the ET gene. A DNA sequence analysis revealed that the ET gene of the ls^1 mutant harbours a 1550 bp deletion which removes the first 185 amino acids of the protein and 865 bp of the sequence which is localized upstream. A second independent mutant allele ls^2 contains a 3 bp insertion and several point mutations in a short DNA portion, one of which results in a termination of the protein after 24 amino acids. The complementation experiments and isolation and mapping of

the cDNAs as well as the sequence analyses of the ET gene from the wild type and two independent *ls* alleles revealed that the cDNA clone ET represents the entire coding sequence of the mRNA of the *ls* gene.

To address the question whether similar or homologous genes are present also in other plant species the cDNA clone ET was employed as hybridization probe in Southern experiments under reduced stringency. The term "plant", as used herein, comprises monocotyledonous and dicotyledonous economic and ornamental plants. The term "reduced stringency", as used herein, is directed to typical hybridization conditions with the modification that hybridization temperature was between 50°C and 55°C. In potato (*Solanum tuberosum*) and snapdragon (*Antirrhinum majus*) several DNA fragments could be detected. From snapdragon several genomic clones were isolated by hybridization at 55°C. A DNA sequence analysis revealed that the isolated snapdragon clone has significant sequence homologies to the *Ls* gene. Thus, genes homologous to the tomato *Ls* gene may be isolated according to conventional methods by using the cDNA clone ET as a probe. Using gene specific primers the *Ls* homologous gene was isolated from genomic DNA of potato (*Solanum tuberosum*) via PCR. The *Ls* homologous gene from potato shows a sequence identity of approx. 98% to the *Ls* gene of tomato on the DNA level as well as on the protein level. From genomic DNA of Arabidopsis (*Arabidopsis thaliana*) a 687 bp DNA fragment of the *Ls* homologous gene was isolated via PCR using degenerate primers. On DNA level the *Arabidopsis thaliana* DNA fragment exhibits a sequence identity of about 63% to the tomato *Ls* gene. On protein level about 55% of the amino acids are identical.

The present invention is further directed to DNA sequences which are derived from a plant genome and code for a protein necessary for controlling side-shoot formation and/or petal formation and/or formation of abscission zones. Upon introduction and expression in plant cells the information contained in the nucleotide sequence results in the formation of a ribonucleic acid. By means of said ribonucleic acid a protein activity may be introduced into the cells or an endogenous protein activity may be suppressed. Particularly preferred is a DNA sequence according to SEQ ID NO: 1 from *Lycopersicon esculentum* shown in Figure 5, a DNA sequence according to SEQ ID NO: 9 from *Solanum tuberosum* shown in Figure 6 and a DNA sequence according to SEQ ID NO: 13 from *Arabidopsis thaliana* shown in Figure 7.

Moreover, the present invention relates to the use of the DNA sequences or fragments or derivatives according to the present invention which are derived from said DNA sequences by insertion, deletion or substitution in the transformation of plant cells. The DNA sequences according to the present invention may be employed using different methods to suppress the formation of side-shoots and thus of branches of the shoot system and/or petals and/or abscission zones:

1. To suppress the formation of side-shoots and/or petals and/or abscission zones the DNA sequence according to the present invention may be cloned in an antisense or a sense orientation into conventional vectors (e.g. plasmids) and thus combined with control elements for expression in plant cells, such as promoters and terminators. By using the prepared vectors, plant cells may be transformed with the aim to prevent the synthesis of the endogenous protein. For this purpose, shorter parts of the DNA sequence according to the invention, i.e. fragments, or DNA sequences having a sequence similarity of from 50% to 100%, i.e. derivatives, may also be used. Thus, the *Ls* homologous gene isolated from Arabidopsis may be employed for example to suppress the formation of side-shoots and thus of branches of the shoot system and/or petals and/or abscission zones in the related species *Brassica napus* (rape). The targeted suppression of a genetic activity in plant cells by the introduction of antisense or sense constructs is a common method which has been successfully employed in many cases (Gray *et al.*, 1992, Plant. Mol. Biol., 19: 69-87).

2. Furthermore, the formation of side shoots and/or petals and/or abscission zones may be inhibited by expressing a ribozyme constructed for this purpose using the DNA sequences according to the present invention. Preparation and use of ribozymes are disclosed in de Feyter *et al.*, 1996, Mol. Gen. Genet., 250: 329-338 for tobacco mosaic virus resistant tomato and tobacco plants.

3. Furthermore, the DNA sequence according to the present invention may be used to inactivate the endogenous gene. By using the DNA sequences of the present invention oligonucleotides may be synthesized to test plants in the context of mutagenesis experiments by means of PCR technique for the presence of insertions (e.g. transposable elements or T-DNA from *Agrobacterium tumefaciens*) in the *Ls* gene. Generally, the genetic activity will be blocked by such insertions (Koes *et al.*, 1995, Proc. Natl. Acad. Sci. USA, 92: 8149-8153).

4. The DNA sequence according to the invention may be also employed to switch off ("knock-out") the endogenous *Ls* gene by means of homologous recombination. This method was successfully employed in mice and is also described for use in plants by Miao and Lam, 1995, Plant. J., 7, 359-365.

In contrast to tomato and other economic plants, in ornamental plants (e.g. geraniums, fuchsias and chrysanthemums) phenotypes are often preferred which exhibit a bushy growth due to a strong development of the side shoots. In order to generate said growth forms today, the plants are either decapitated, which promotes the initiation of side axes, or are treated with particular chemicals. However, said practice is also associated with considerable costs. In these cases, the preparation of transgenic plants having bushy growth forms according to the present invention represents a more cost-effective alternative.

In ornamental plants an enhanced formation of abscission zones my be used such that after fading the flowers fall off by themselves and must not be manually removed as with many balcony and garden plants. If this does not occur, the formation of new flowers often is suppressed.

For the preparation of transgenic plants with strong side-shoot formation and/or abscission zone formation the DNA sequence or fragment or derivative thereof according to the invention which is derived from said sequence by insertion, deletion or substitution, is introduced into plasmids in a sense orientation and combined with control elements for expression in plant cells. Using said plasmids plant cells may be transformed such that a translatable messenger ribonucleic acid (mRNA) is expressed which enables the synthesis of a protein stimulating the formation and development of side shoots and/or petals and/or abscission zones.

The DNA sequence or fragments or derivatives thereof according to the present invention which are derived from said sequence by insertion, deletion or substitution may be used to isolate homologous or similar DNA sequences from the genome of tomato or other plants, which DNA sequences influence the formation of side shoots and/or petals and/or abscission zones as well. For this purpose the DNA sequence or fragments, e.g. oligonucleotides, or derivatives according to the present invention may be employed as probe molecules to screen cDNA libraries or genomic DNA libraries of the plants to be screened according to conventional methods. Alternatively, degenerated

or non-degenerated oligonucleotides (primers) may be derived from the sequence according to the present invention, which may be used to screen said cDNA libraries or genomic DNA libraries on a PCR basis. Similar to the DNA sequences according to the present invention, the thus isolated related DNA sequences may be employed for inhibition or stimulation of side-shoot formation and/or petal formation and/or abscission zone formation in plants.

For expression of the DNA sequences according to the present invention in sense or antisense orientation in plant cells on the one hand transcription promoters and on the other hand transcription terminators are necessary. A great number of promoters and terminators have been described in the literature (e.g. Köster-Töpfer *et al.*, 1989, Mol. Gen. Genet., 219: 390-6; Rocha-Sosa *et al.*, 1989, EMBO J., 8: 23-29). The transcriptional initiation and termination regions may be derived either from the host plant or from a heterologous organism. The DNA sequences of the transcription initiation and transcription termination regions may be prepared synthetically or obtained naturally or may contain a mixture of synthetic and natural DNA components.

Methods for genetic modification have been described for dicotyledonous and monocotyledonous plants (Gasser and Fraley, 1989, Science 244: 1293-1299; Potrykus, 1991, Ann. Rev. Plant. Mol. Biol. Plant. Physiol., 42: 205-226). In addition to the transformation by means of *Agrobacterium tumefaciens* (Hoekema, 1983, Nature, 303: 179-180; Filatti *et al.*, 1987, Biotech, 5:726-730), DNA may be introduced by transformation of protoplasts, microinjection, electroporation or ballistic methods into plant cells. For selection of transformed plant cells the DNA to be introduced is coupled with a selection marker which imparts resistance against antibiotics (e.g. kanamycin, hygromycin, bleomycin) to the cells. From the transformed plant cells whole plants may then be regenerated in a typical selection medium. Regeneration of plant cells is described for example in EP-B-0 242 236, which is incorporated herein by special reference. The plants thus obtained are tested for the presence and intactness of the introduced DNA by means of conventional molecular biological methods. Once the introduced DNA is integrated into the genome, it is generally stable and is transmitted to the offspring. By using conventional methods seed stocks may be obtained from the resulting plants.

The following examples are meant to illustrate the present invention and are not construed to be limiting. If not mentioned otherwise, molecular biological standard procedures were used, as described by Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. Southern hybridizations were carried out in 6 x SSPE (0.9 M NaCl, 50 mM NaH_2PO_4 x H_2O, 5 mM EDTA, 0.1% BSA, 0.1% Ficoll, 0.1% PVP, 0.5% SDS, 100 µg/ml of calf thymus DNA) with a Hybond N+ membrane (Amersham). Plaque hybridizations were performed in 6 x SSPE (1.08 M NaCl, 60 mM NaH_2PO_4 x H_2O, 6 mM EDTA, 0.1% BSA, 0.1% Ficoll, 0.1% PVP, 0.1% SDS, 200 µg/ml of calf thymus DNA) with a Hybond N+ membrane (Amersham).

Example 1

Isolation of YAC clones from the *Ls* region of tomato

From a tomato YAC library (Martin *et al.*, 1992, Mol. Gen. Genet., 233: 25-32) clones were isolated containing CD61 marker (Schumacher *et al.*, 1995, Mol. Gen. Genet., 246: 761-766). For this, DNA mixtures which were derived from a microtiter plate with 96 YAC clones were first tested by using the conventional PCR method. Thus, from 144 of such DNA mixtures nine could be identified which yielded a PCR product with the CD61-F and CD61-R primers (Schumacher *et al.*, 1995, Mol. Gen. Genet., 246: 761-766). The isolation of single clones was carried out by means of colony hybridization or PCR, wherein the DNA of clones of a row or column of a microtiter plate was used as a mixture. Thus, from 96 clones of a plate single clones were identified using 20 PCR reactions. In total, five YAC clones were identified, the insert size of which was determined to be 280 – 320 kb by pulsed field gel electrophoresis (Chu *et al.*, 1986, Science, 234: 1582-1585). It was shown in PCR and Southern experiments that YAC CD61-5, in addition to CD61, also carried the second flanking marker CD65 and thus spanned the *Ls* locus.

Example 2

Isolation of cDNA clones of the *Ls* region from tomato

For preparation of a hybridization probe DNA from the YAC clone CD61-5 was isolated following separation by means of pulsed field gel electrophoresis. However,

separation on said pulsed field gel only allowed for a relatively rough preparation, such that the probe used, in addition to the YAC clone CD61-5, also contained portions of the DNA from yeast chromosome III (360 kb) and VI (280 kb). Following radio-labeling said DNA was used as a probe to screen 5 x 10^5 pfu (plaque forming units) in a conventional plaque hybridization. Hybridization with the YAC probe provided a plurality of signals of different intensity. For rescreening 50 plaques of different signal intensities were selected and 44 purified clones could then be grouped by means of cross hybridization. 23 of 44 clones which resulted from rescreening were present only once. In total, 29 different transcripts were identified in this screening. Following establishment of a cosmid contig the cDNA library was again screened with the cosmid clones to isolate additional cDNA clones which were not detectable in screening with YAC61-5 as a probe due to the high complexity of the probe. In these experiments, three additional cDNA clones were isolated. In total 32 different transcripts were detected.

Example 3

RFLP mapping of isolated cDNA clones from tomato

Of 30 identified transcripts 22 showed typical hybridization patterns for single or low-copy sequences which enabled RFLP mapping. In a first RFLP analysis the isolated cDNA clones were hybridized against filters which carried DNA from *L. esculentum*, *L. penellii* as well as from the back crossing line IL83 digested with the restriction endonuclease enzymes EcoRI, EcoRV and XbaI (Eshed *et al.*, 1992, Theor. Appl. Genet., 83: 1027-1034). This line, in which the distal terminus of chromosome 7 is derived from *L. pennellii* while the rest of the genome is composed of *L. esculentum* chromosomes, enables a first rough mapping in the presence of a polymorphism between *L. esculentum* and *L. pennellii*. If a polymorphous DNA fragment was derived from the *Ls* region, the line IL83 exhibited the *L. pennellii* allele, whereas the *L. esculentum* allele was present for fragments from the remaining genome. In this manner four cDNA clones were identified which were not derived from chromosome 7. Fine mapping of the 18 remaining cDNA clones derived from chromosome 7 was carried out via RFLP analysis of the plants W23 and W24 which contained recombination events in the interval CD61-*Ls* and *Ls*-CD65, respectively. Since in this analysis candidates for

the *Ls* gene in plant W23 exhibited the *L. esculentum* as well as the *L. pennellii* specific fragment, while in plant W24 only the *L. esculentum* specific fragment was present, the cDNA clones were hybridized against filters carrying genomic DNA digested with EcoRI, EcoRV or XbaI of both parental species as well as of both recombinants W23 and W24. In this manner a total of four cDNA clones was identified which cosegregated with the *Ls* gene and thus, were possible candidates for the *Ls* gene.

Example 4

Preparation and screening of a genomic cosmid library of tomato

DNA of the T-DNA/cosmid vector pCLD04541 (Bent *et al.*, 1994, Science, 265: 1856-1860) was isolated according to the protocol of Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, purified via two CsCl gradients and dialyzed against TE for 3 days. The DNA was completely digested with BamHI and subsequently dephosphorylated with alkaline phosphatase to prevent self ligation of the vector. 200 ng of genomic tomato DNA partially digested with MboI and 2 mg of vector DNA were ligated with T4 DNA ligase in 10 ml at 16°C over night. 3 ml of said ligation assay were employed for packaging and transfected into *E. coli* SURE (Stratagene). This assay resulted in 6 x 10^6 independent recombinant bacteria. Each of 100 plates were plated with 2500 cfu (colony forming units) and rinsed off with 10 ml each of LB medium. In each case a glycerol culture was made from this material and a DNA preparation was carried out. These 100 DNA pools were screened by means of PCR analysis. Positive pools were then subjected to colony filter hybridization to identify positive single clones.

Example 5

Cloning and sequencing of the *Ls* gene from tomato

The insert of the cDNA clone ET which was isolated as a probe in screening of the cDNA library with cosmid G was cut out with EcoRI and cloned into vector pGEM-11Zf(+). The missing 5' terminus of the gene was isolated by means of the RACE technique (Frohman *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85: 8998-9002). Here, starting from an oligonucleotide specifically binding to known regions of the gene, a

DNA complementary to RNA (cDNA) was prepared. Subsequently deoxycytosin nucleotides were attached to the cDNA using terminal transferase. With a second gene specific primer and a primer binding to the polydeoxycytosin tail the 5' end of the cDNA was amplified via PCR and cloned into the plasmid vector pGEM-T. Subsequently the longest of the RACE clones were sequenced. Simultaneously with the analysis of cDNA clone ET subfragments of the respective genomic region of cosmid G were isolated and recloned into the plasmid vectors pGEM-4Z and pSPORTI. Overlapping subfragments were then sequenced. The genomic sequence did not show any difference from the sequence of the cDNA clone, which means that the Ls gene does not contain any intron. Moreover, the respective genome regions of both mutants ls^1 and ls^2 were amplified from the genomic DNAs via PCR using suitable primers and cloned into the pGEM-T vector. Sequence analysis of said products exhibited a deletion of 1.5 kb in the ls^1 allele compared to the wild type sequence. Besides the loss of nucleotides 1-685 of the open reading frame the ls^1 mutant also lacks 865 base pairs of the region located 5' of the open reading frame, which is thought to have a regulatory function (promoter) for expression. Therefore, it may be assumed that the ls^1 mutant is no longer able to form a functional protein from the *Ls* gene. In the ls^2 allele an insertion of 3 base pairs as well as 3 base exchanges were found in the 5' region of the open reading frame. One of these base exchanges leads to a stop codon resulting in a termination of the amino acid chain after 24 amino acids. Again a protein without any function is to be assumed. The vectors pGEM-11zf(+), pGEM-4z, pGEM-T were purchased from the company Promega Corp., Madison, U.S.A., vector pSPORTI was purchased from the company Life Technologies, Eggenstein, and used according to the manufacturer's instructions.

Example 6

Transformation of plants with *Ls* cDNA constructs of tomato

Ls cDNA was isolated with gene specific primers CD61-13 (5'-TTAGGGTTTTCACTCCACGC-3'; SEQ ID NO: 3) and CD61-28 (5'-TCCCCTTTTTTTCCTTTCTCTC-3'; SEQ ID NO: 4) by means of the conventional PCR method and cloned into plasmid vector pGEM-4z (GSET8). For preparation of the transformation constructs the *Ls* cDNA was cut off from plasmid GSET8 with SalI/SstI (for sense construct) and XbaI/SstI (for antisense construct) and ligated into the plant

transformation vector pBIR digested with SalI/SstI (sense construct) and XbaI/SstI (antisense construct), respectively (Meissner, 1990, doctoral thesis, University of Cologne, Cologne). In the resulting clones the cDNA is present either in sense or in antisense orientation between promoter and polyadenylation site of the 35S gene of cauliflower mosaic virus. The resulting sense and antisense plasmids were introduced into the *Agrobacterium tumefaciens* strain GV3101 (Koncz and Shell *et al.*, 1986, Mol. Gen. Genet., 204: 383-396) by direct transformation. Subsequently the T-DNAs of the two different constructs were transformed into leaf pieces of tomato and tobacco according to Fillatti *et al.*, 1987, Biotech, 5: 726-730. Different transgenic plants containing the *Ls* antisense construct show a reduction of side-shoot formation

Example 7

Isolation of a *Ls* related gene from snapdragon (*Antirrhinum majus*)

With cDNA clone ET as a probe a genomic phage library from *Antirrhinum majus* was screened. Hybridization was carried out at 55°C, i.e. under reduced stringency. In this experiment 14 clones were isolated, clone HH13 of which showing the strongest hybridization signals was further characterized. The sequence analysis carried out following recloning the phage insert into the plasmid vector pGEM-11zf(+) showed that the isolated *Antirrhinum majus* gene has high sequence homology to the *Ls* gene from tomato. Within both sequences regions could be identified, in which the derived amino acid sequence is totally conserved.

Example 8

Isolation of an *Ls* related gene from potato (*Solanum tuberosum*)

In a Southern blot experiment under reduced stringency at 55°C using cDNA of the *Ls* gene as a hybridization probe, a DNA fragment could be detected in genomic DNA from *Solanum tuberosum* (Fig. 4). Using gene specific primers CD61-24 (5'-TTTCCCACTCAAGCCAACTC-3'; SEQ ID NO: 5), CD61-6 (5'-GGTGGCAATGTAGCTTCCAG-3'; SEQ ID NO: 6), PO1 (5'-TCGAGGCGTTGGATTATTATAC-3'; SEQ ID NO: 7) and PO5 (5'-GGCCCCCATATCTTTTTCC-3'; SEQ ID NO: 8) from *Ls* gene overlapping genomic DNA fragments were isolated from conventionally isolated DNA from *Solanum*

tuberosum by using the PCR method. The PCR reactions were carried out as follows: Denaturation at 95°C for 30 seconds, annealing at 60°C for 1 minute, elongation at 72°C for 2 minutes. This cycle was repeated 30 times. The resulting PCR products were cloned into the plasmid vector pGEM-T. A sequence analysis revealed that the isolated DNA fragments from *Solanum tuberosum* bear the sequence information for an open reading frame having a coding capacity of 431 amino acids (Fig. 6). The DNA sequence is shown in SEQ ID NO: 9 and the amino acid sequence encoded by the DNA sequence is illustrated in SEQ ID NO: 10. On DNA level as well as on protein level the Ls homologous gene of potato exhibits a sequence identity of about 98% to the *Ls* gene of tomato.

Example 9

Isolation of an *Ls* related gene from *Arabidopsis thaliana*

For the isolation of the *Ls* homologous gene from *Arabidopsis thaliana* the degenerated primers CD61-38 (5'-CARTGGCCNCCNYTNATGCA-3'; SEQ ID NO: 11)* and CD61-41 (5'-TGRTTYTGCCANCCNARRAA-3'; SEQ ID NO: 12)* were made and used for PCR reactions with genomic DNA from *Arabidopsis thaliana* isolated in a usual manner. The PCR reactions were carried out as follows: Denaturation at 95°C for 30 seconds, annealing at 50°C for 1 minute, elongation at 72°C for 1 minute. This cycle was repeated 35 times. In this manner a DNA fragment of about 700 bp could be amplified which was subsequently cloned into the plasmid vector pGEM-T. A sequence analysis showed that the isolated DNA fragment from *Arabidopsis thaliana* (SEQ ID NO: 13) was 687 bp in length and has a high sequence similarity to the *Ls* gene from *Lycopersicon esculentum*. On the DNA level the *Arabidopsis thaliana* DNA fragment shows a sequence identity of about 63% to the *Ls* gene of tomato. On the protein level about 55% of the amino acids are identical The amino acid sequence encoded by the isolated DNA fragment (SEQ ID NO: 13) is illustrated in SEQ ID NO: 14. By using the isolated DNA fragment *the* Ls homologous gene from *Arabidopsis thaliana* may be isolated using conventional molecular biological standard methods.

* In the description of the degenerated primers the WIPO standard St. 23 was used:

$R = A + G$

$N = A + G + C + T$

$Y = C + T$